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Myogenin and MyoD join a family of skeletal muscle genes regulated by electrical activity

(denervation/transcription/differentiation/innervation)

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ABSTRACT **Myogenin and MyoD are proteins that bind to the regulatory regions of a battery of skeletal muscle genes and can activate their transcription during muscle differentiation. We have recently found that both proteins interact with the enhancer of the nicotinic acetylcholine receptor (nAChR) α subunit, a gene that is regulated by innervation. This observation prompted us to study if myogenin and MyoD transcript levels are also regulated by skeletal muscle innervation. Using Northern blot analysis, we found that MyoD and myogenin mRNA levels begin to decline at embryonic day 17 and attain adult levels in muscle of newborn and 3-week-old mice, respectively. In contrast, nAChR mRNAs are highest in newborn and 1-week-old mouse muscle and decline thereafter to reach adult levels in 3-week-old mice. To determine if the down-regulation of myogenin and MyoD mRNA levels during development is due to innervation, we quantitated message levels in adult calf muscles after denervation. We found that in denervated muscle myogenin and MyoD mRNAs reach levels that are approximately 40- and 15-fold higher than those found in innervated muscle. Myogenin mRNAs begin to accumulate rapidly between 8 and 16 hr after denervation, and MyoD transcripts levels begin to increase sharply between 16 hr and 1 day after denervation. The increases in myogenin and MyoD mRNA levels precede the rapid accumulation of nAChR α-subunit transcripts; receptor mRNAs begin to accumulate significantly after 1 day of denervation. The effects of denervation are specific because skeletal α-actin mRNA levels are not affected by denervation. In addition, we found that the repression of myogenin and MyoD expression by innervation is due, at least in part, to "electrical activity." Direct stimulation of soleus muscle with extracellular electrodes repressed the increase of myogenin and MyoD transcripts after denervation by 4- to 3-fold, respectively. In view of these results, it is interesting to speculate that myogenin and/or MyoD may regulate a repertoire of skeletal muscle genes that are down-regulated by electrical activity.**

Development of skeletal muscle cells is characterized by a series of events that include commitment, differentiation, and maturation. Myoblasts arise from the commitment of pluripotential mesodermal cells to the myogenic lineage. The myoblasts proliferate and later differentiate and fuse to form multinucleated embryonic myotubes. Differentiation is characterized by the transcriptional activation of a battery of muscle-specific genes coding for metabolic enzymes, contractile proteins, ion channels, and neurotransmitter receptors (1–4). Terminal differentiation continues as embryonic myotubes become innervated by motoneurons during a period known as maturation. There are selective changes that occur in skeletal muscle fibers during maturation. Innervation down-regulates a subset of skeletal muscle synaptic proteins, which include nicotinic acetylcholine receptors (nAChRs) (4), voltage-gated sodium channels (2, 3), and adhesion molecules (5), but does not modify the expression of other enzymes or structural proteins, such as creatine kinase, myosin light chain, and skeletal α-actin. The selective regulation of proteins and their mRNAs during innervation can result from the transcriptional regulation of genes that respond to chemical cues or electrical signals sent by the nerve.

Important progress has recently been made in identifying the DNA regulatory sequences and factors that interact to regulate transcription of skeletal muscle genes. A family of muscle-specific regulatory factors, containing a region bearing homology to the *myc* oncogene, has been recently characterized (6–10). Two members of the family, myogenin and MyoD, have been shown to be nuclear proteins (11, 12) that can bind to regulatory elements of muscle genes. Transfection of nonmuscle cells with myogenin or MyoD cDNAs leads to the transcriptional activation of a battery of skeletal muscle genes and commits the cells to become myoblasts (6–10). Myogenin and MyoD may have important functions in later development since they have been shown to bind directly to the enhancers and to activate transcription of the creatine kinase (9, 12, 13) and myosin light chain (N. Rosenthal, personal communication; ref. 13) genes. Both genes are activated in skeletal muscle during myoblast differentiation.

We were interested in analyzing if myogenin and MyoD may also function during muscle maturation, since we have recently found that both proteins bind to the nAChR α-subunit enhancer, and their cDNAs transactivate the expression of a reporter gene placed under the control of the α-subunit upstream regulatory elements (A.B., unpublished data). The fact that nAChR genes, as well as other genes coding for synaptic proteins, are initially expressed during myoblast differentiation but are later down-regulated by innervation prompted us to study if the expression of myogenin and MyoD is regulated during muscle maturation. Furthermore, we analyzed myogenin and MyoD mRNA levels after muscle denervation. Responses of muscle to denervation can indicate that innervation modulates processes in the myofiber (14–16). Herewith, we report that during embryonic and perinatal mouse development, myogenin and MyoD mRNA levels are down-regulated by innervation. Denervation of adult muscle leads to a large accumulation of both transcripts. The changes in myogenin and MyoD mRNA levels during innervation, and after denervation, precede the changes of nAChR messages. In addition, we demonstrate that direct stimulation of denervated rat soleus muscle with

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Abbreviations: nAChR, nicotinic acetylcholine receptor; E, embryonic day.

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extracellular electrodes suppresses the increase of myogenin, MyoD, and receptor mRNAs.

MATERIALS AND METHODS

Isolation, Denervation, and Stimulation of Skeletal Muscle. Developmental studies were done using staged embryos; embryonic day 1 (E1) refers to the day after copulation. The muscles dissected from the hindlimbs of embryos from a litter were pooled and considered as a single preparation. The muscles isolated from E15 and E17 mice may have contained minor amounts of bone residue that were difficult to remove during dissection. For denervation studies, the sciatic nerve of anesthetized 8-week-old Swiss mice (Metofane; Pitman-Moore, Washington Crossing, NJ) was unilaterally resected at the upper thigh. At different times after surgery total RNA was isolated from the posterior crural muscle groups. The crural muscles from approximately six mice were pooled for each RNA preparation.

Stimulation of adult soleus rat muscle (Sprague–Dawley) was performed as described (17). Anesthetized animals were denervated by bilateral removal of a 5-mm segment of the sciatic nerve and electrodes were implanted into one of the hindlimbs. The soleus muscle was stimulated chronically for 6 or 10 days in 100-Hz trains, 1-s duration, applied once every 100 s. The train of pulses were of alternating polarity with a strength of 10–15 mA and a duration of 0.5 ms. The contralateral denervated/unstimulated muscle served as a control. After stimulation, the soleus muscles were removed for RNA preparations.

RNA Preparations and Northern Blots. Total RNA was isolated from mouse and rat muscles using the guanidine thiocyanate/cesium chloride ultracentrifugation method (18). RNA was fractionated by electrophoresis on 1.5% agarose gels containing 2.2 M formaldehyde, and then the gels were electroblotted onto Nytran membranes (Schleicher & Schuell). Hybridization was carried out at 42°C in 6× SSC (1× SSC is 0.15 M NaCl/1.5 mM sodium citrate), 10× Denhardt's solution (1× is 0.02% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin), 1% SDS, 10 mM EDTA, 0.1% sodium pyrophosphate, 100 μg of salmon sperm DNA per ml, and either 50% or 40% formamide (when using the mouse MyoD and nAChR probes on rat RNA). Blots were washed with 0.1× SSC/1% SDS at 65°C, or with 0.2× SSC/1% SDS at 60°C when using heterologous probes. Initially, the blots were exposed to x-ray film to obtain an autoradiogram, and subsequently, the hybridizing probes were quantitated directly from the blots (see below). The cDNA probes were ^{32}P-labeled by random priming (19) to a specific activity of $\approx 10^8$ cpm/μg of DNA. The probes used for hybridization were an *Eco*RI 1.7-kilobase (kb) mouse MyoD cDNA insert (6), two fragments of 1.0 and 0.5 kb generated by *Eco*RI digestion of a mouse myogenin cDNA (9), an *Eco*RI 1.5-kb fragment of a rat myogenin cDNA (8), an *Eco*RI 1.6-kb insert of a mouse nAChR α-subunit cDNA (20), and a plasmid containing sequence of a partial mouse skeletal α-actin cDNA (21). To minimize experimental variability the blots were stripped after quantitation and used for hybridization with other probes. The blots were stripped in 60% formamide/1% SDS/10 mM Tris·HCl, pH 7.5, at 65°C, and exposed to x-ray film to assert that the probe was fully stripped.

Quantitation of Relative mRNA Levels on Northern Blots. The amount of radioactivity on the blots associated with the different bands was quantitated directly by using an Ambis radioanalytical imaging system (Ambis Systems, San Diego) or a Betascope 603 blot analyzer (Betagen, Waltham, MA). The amount of radioactivity per band varied linearly with respect to time and with respect to the amount of mRNA on the blots (data not shown). The backgrounds obtained on each blot were subtracted.

RESULTS

Myogenin and MyoD mRNAs Are Down-Regulated by Innervation. Recently, Sassoon *et al.* (22) used *in situ* hybridization to analyze the expression of myogenin and MyoD mRNAs during early mouse myogenesis. They observed that the transcripts coding for both proteins are initially expressed asynchronously in somites and that by 11.5 days *post coitum*, both transcripts accumulate synchronously in mononucleated cells of the forelimb and hindlimb buds. These studies, however, did not extend to later stages of development when secondary embryonic myotubes are formed and innervated. To evaluate if myogenin and MyoD levels are regulated by innervation, we analyzed the expression of their transcripts during development (Fig. 1) and after denervation (Fig. 2) using Northern blot analysis. RNA isolated from the hindlimb muscles of E15 to 8-week-old mice was used for the developmental studies. The results obtained using blots hybridized with myogenin, MyoD, and nAChR α-subunit cDNA probes are shown in Fig. 1. The signals from the blots were quantitated (see *Materials and Methods*) and plotted as a function of development on Fig. 3*A*. The transcript levels for myogenin and MyoD drop during muscle maturation. Initially, myogenin mRNA levels increase between E15 and E17 and begin to decrease thereafter. Between E17 and the third week of postnatal development, the quantities of myogenin tran-

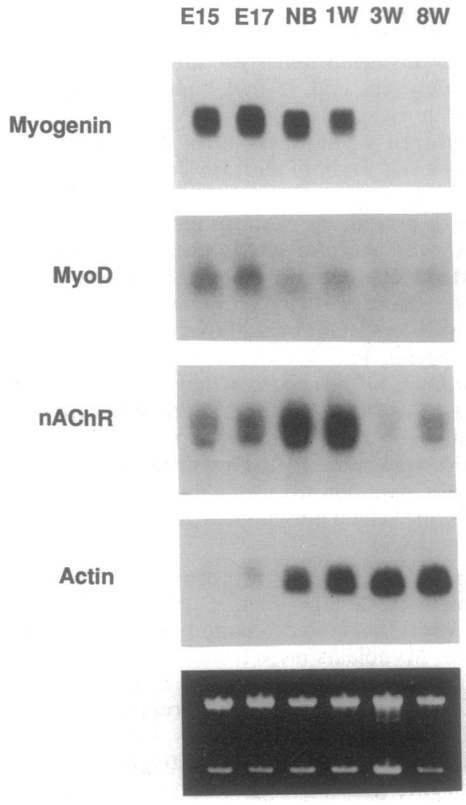

FIG. 1. Myogenin and MyoD mRNA levels are down-regulated during development. The lanes in the representative blot shown above contain 10 μg of total RNA. RNA was quantitated spectrophotometrically, and the integrity and relative amounts of RNA in each sample used were checked by ethidium bromide staining of rRNA on a separate gel (bottom panel). The probes used were cDNA fragments coding for mouse myogenin (9), mouse MyoD (6), mouse nAchR α subunit (20), and mouse skeletal actin (21). The blots were exposed to x-ray film plus intensifying screens for 36–84 hr. NB, newborn; W, week(s).

scripts decrease by 15-fold and reach adult levels. MyoD mRNA levels are dropping by E15 and attain adult levels by birth (Fig. 3*A*). Although myogenin and MyoD transcript levels markedly decline during skeletal muscle maturation, the signals obtained with RNA from adult mice were consistently higher than background. The differences in myogenin mRNA levels found between fetal and adult muscle are similar to those observed by Wright *et al.* (8).

The blots were also probed for the mouse nAChR α-subunit mRNAs. Several studies have described the repressive effects of innervation on the expression of nAChRs (reviewed in ref. 4) and receptor subunit mRNAs (15, 16). As shown in Figs. 1 and 3*A*, receptor mRNA levels increase in muscles from E17 to 1-week-old mice and thereafter begin to decline. Adult levels of receptor mRNA are attained by the third week of postnatal development. Blots hybridized with a mouse nAChR δ-subunit probe gave comparable results (data not shown). It is interesting to note that during development myogenin and MyoD transcript levels begin to decrease before receptor mRNAs drop. The diminution of myogenin, MyoD, and nAChR mRNA expression during skeletal muscle maturation is selective, because α-actin mRNAs increase gradually from E17 to adulthood and are not down-regulated by innervation (Fig. 1).

Denervation Induces the Expression of Myogenin and MyoD Transcripts. The expression of genes coding for several synaptic proteins, including nAChRs, is initially activated during myoblast differentiation and later is strongly down-regulated by innervation. Denervation, however, reverses the repressive effects of innervation and results in the reaccumulation of mRNAs coding for synaptic proteins (23). To assess if innervation down-regulates the expression of myogenin and MyoD, we measured the levels of both transcripts after denervation. The sciatic nerves of adult mice were transected, and at different times after denervation total RNA was isolated from the posterior crural muscle groups for Northern blot analysis. The blots were hybridized with cDNAs coding for myogenin and MyoD. As controls, the blots were also hybridized with the nAChR α-subunit and skeletal α-actin cDNA probes (Fig. 2). The radioactivity in the hybridizing bands of the blots was quantitated directly and the results are plotted in Fig. 3*B*. Myogenin and MyoD transcripts begin to rapidly accumulate after 8 and 16 hr postdenervation, respectively. In all of the experiments myogenin mRNAs attained their highest levels by 2 days after denervation; the levels are ≈40-fold higher than those measured in innervated muscle (Fig. 3*B*). MyoD transcripts begin to accumulate steadily after 16 hr of denervation, and by 1 week after surgery, the levels are 15-fold higher than those of innervated muscle. The changes in nAChR α-subunit messages after denervation were also measured. The levels of receptor mRNAs begin to accumulate significantly after 1 day of denervation, and by day 7 they are ≈70-fold higher than in

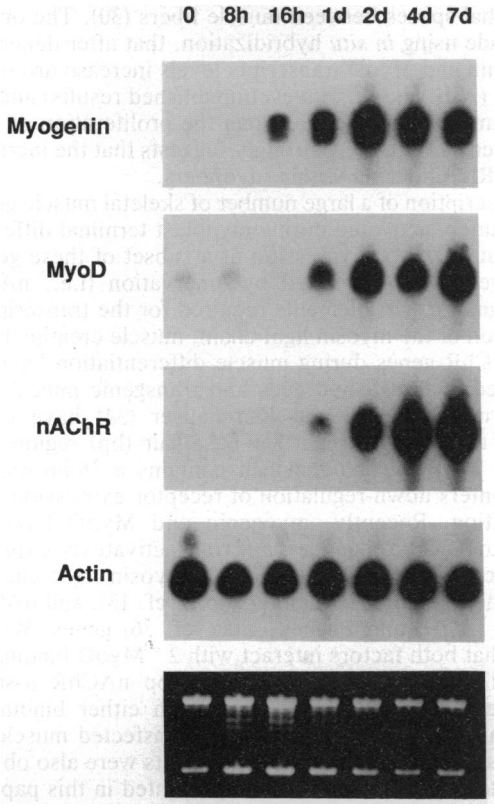

FIG. 2. Myogenin and MyoD transcripts accumulate after denervation of skeletal muscle. Northern blots containing 10 μg of total RNA isolated from innervated or denervated adult mice muscles were hybridized with myogenin, MyoD, nAChR α-subunit, and mouse α-actin probes. Posterior crural muscles were dissected from 8-week-old mice (0) or mice denervated for 8 hr, 16 hr, 1 day, 2 days, 4 days, and 7 days.

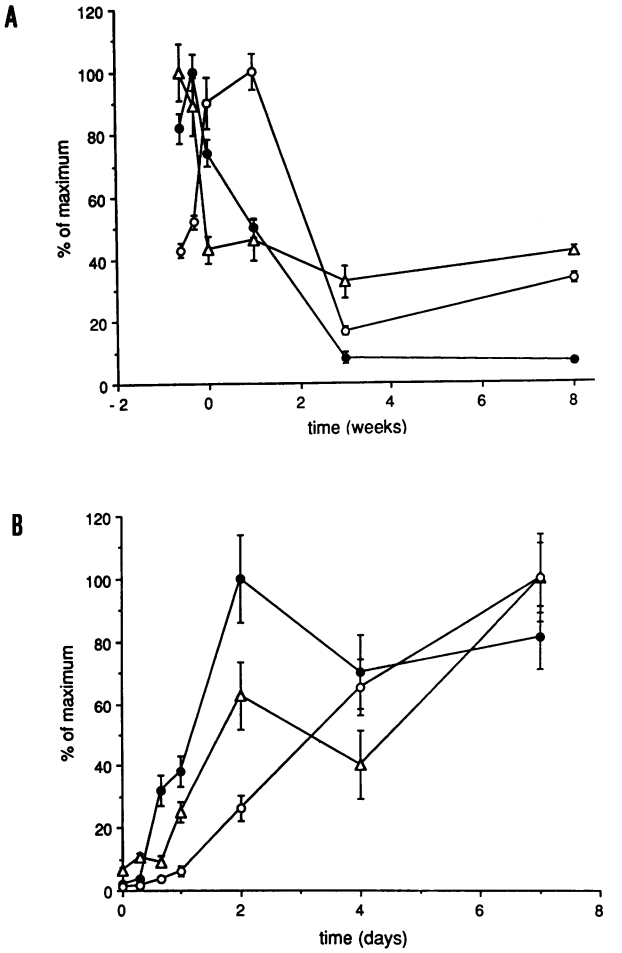

FIG. 3. Quantitation of the relative amounts of myogenin and MyoD transcripts during development and after denervation. The relative levels of myogenin (●), MyoD (△), and nAChR α-subunit (○) transcripts were quantitated during development (*A*) and at different times after denervation (*B*). The procedure used to quantitate the signals directly from the blots is described in the text. We verified that the radioactivity obtained increased linearly with respect to counting time and amounts of RNA loaded on the gels. Each point represents the mean ± SEM ($n = 3$). The values are expressed as the % of the maximum signal. Points at time "0" represent RNA isolated at birth (*A*). The data in *B* are normalized to skeletal α-actin signals to account for any muscle degeneration that may have occurred during the denervation time course.

innervated muscle. The increases in myogenin, MyoD, and nAChR transcripts after denervation are specific because the levels of α-actin mRNA remained constant for most of the time course. The slight decrease in actin mRNAs at day 7 (1.3-fold) may result from the gradual atrophy of muscle after nerve resection. It is interesting to speculate that myogenin and/or MyoD may modulate the expression of a large repertoire of genes regulated by innervation, since changes in their mRNA levels precede the changes in receptor message during muscle maturation and after denervation.

Electrical Activity Regulates Myogenin and MyoD mRNA Levels. Innervation may regulate muscle properties by sending chemical signals that are released by the nerve or located on its surface (24, 25) and by the "electrical activity" resulting from muscle depolarization during neuromuscular transmission (26). To test if electrical activity *per se* down-regulates the levels of myogenin and MyoD transcripts, electrodes were implanted into the hindlimbs of rats immediately after denervation and electrically stimulated (see *Materials and Methods*) for 6 or 10 days. After stimulation, the denervated/stimulated and contralateral denervated/unstimulated soleus muscles of each rat were processed separately to isolate RNA for Northern blots. Blots containing equal amounts of total RNA were probed with cDNAs coding for the myogenic factors and the nAChR α subunit. As shown in Fig. 4*A*, direct stimulation of denervated muscle with extracellular electrodes suppresses the accumulation of myogenin and MyoD mRNAs. Blots containing the RNA isolated from rats stimulated for 6 days (4 rats) and 10 days (2 rats) were quantitated; the results are presented in Fig. 4*B*. The myogenin and MyoD mRNA levels in the denervated/stimulated muscles were on average ≈23% and ≈33% of those found in the contralateral denervated/unstimulated muscles, respectively. The expression of the nAChR α-subunit mRNA, previously shown to be down-regulated by muscle activity using the same stimulation paradigm (17), was also quantitated. The levels of receptor mRNA in the denervated/stimulated muscles were ≈7% of those found in contralateral denervated/unstimulated muscles. The effect of muscle activity on myogenin, MyoD, and receptor mRNA expression is specific, because the levels of α-actin transcripts in denervated/stimulated muscle were similar, or even higher, to those observed in denervated/unstimulated muscle (data not shown). These experiments demonstrate that electrical activity *per se* can selectively suppress, at least in part, the increases of myogenin and MyoD transcripts caused by denervation.

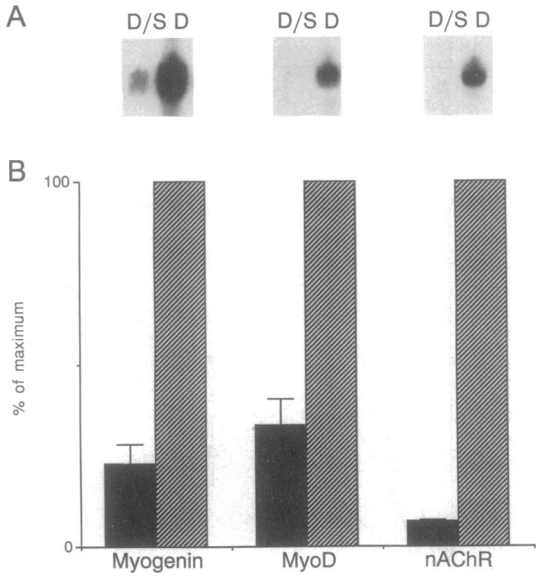

FIG. 4. Electrical activity down-regulates the expression of myogenin and MyoD mRNAs in denervated muscle. (*A*) Representative Northern blots containing 10 μg of total RNA isolated from 6-day denervated/stimulated (D/S) and from the contralateral denervated/unstimulated (D) rat soleus muscles. The blots were hybridized with cDNA probes coding for rat myogenin (8), MyoD, and nAChR α subunit. (*B*) Quantitation of signals obtained on Northern blots. Total RNA was isolated separately from each of six pairs of denervated/stimulated and from contralateral denervated/unstimulated rat soleus muscles, and the signals obtained on the blots were quantitated. To eliminate variability between animals, we calculated the relative radioactivity in each denervated/stimulated sample as compared to the radioactivity from the contralateral denervated muscle, and the ratios obtained from each muscle pair were averaged. The stippled columns represent their mean ± SEM.

DISCUSSION

In the present study we have concentrated on analyzing the expression of myogenin and MyoD mRNAs after skeletal muscle innervation. We found that the expression of both transcripts is repressed during development in the hindlimb muscles of E17 to 8-week-old mice. Myogenin and MyoD transcripts levels begin to decline between E15 and E17 and continue falling until the first to third week of postnatal development. This is a period of development that coincides with innervation of secondary myotubes and the retraction of multiple synapses (27–29). The fact that denervation of adult muscle leads to the rapid accumulation of both transcripts is consistent with the idea that innervation suppresses the expression of myogenin and MyoD. Denervation has multiple effects on muscle; it leads to changes in the biochemical and molecular properties of the myofiber (2–4, 26), and it stimulates the proliferation of mononucleated cells located in the interstitial spaces between muscle fibers (30). The observation made using *in situ* hybridization, that after denervation myogenin and MyoD transcripts levels increase around myonuclei (A.B. and W. Hayes, unpublished results) and begin to accumulate much earlier than the proliferation of mononucleated cells (30, 31), strongly suggests that the increase of both mRNAs occurs within myofibers.

Transcription of a large number of skeletal muscle genes is coordinately activated during myoblast terminal differentiation, but later, the expression of a subset of these genes is selectively down-regulated by innervation (i.e., nAChR). The regulatory cis elements required for the transcriptional activation of the myosin light chain, muscle creatine kinase, and nAChR genes during muscle differentiation have been identified in transfected cells and transgenic mice (32–34). Furthermore, Merlie and Kornhauser (34) have demonstrated that the upstream 850-base-pair (bp) region of the nAChR α-subunit gene, which contains a 36-bp enhancer (35), confers down-regulation of receptor expression during innervation. Recently, myogenin and MyoD have been shown to bind the enhancers and transactivate the expression of the creatine kinase (9, 12, 13), myosin light chain (N. Rosenthal, personal communication; ref. 13), and nAChR α subunit (A.B., unpublished data; ref. 36) genes. We have found that both factors interact with 2 "MyoD binding sites (CANNTG)" located within the 36-bp nAChR α-subunit enhancer and that point mutations in either binding site practically abolish transcription in transfected muscle cells (A.B., unpublished data). Similar results were also obtained by Piette *et al.* (36). The results presented in this paper are consistent with the idea that repression of either myogenin and/or MyoD expression during skeletal muscle development can account for the down-regulation of nAChR genes by innervation. Several lines of evidence support this idea: (*i*) changes in the levels of myogenin and MyoD transcripts precede the changes in receptor mRNAs during development and after denervation, (*ii*) direct stimulation of denervated

muscle *in vivo* selectively suppresses the increase of myogenin, MyoD, and nAChR mRNA levels, (*iii*) both factors bind and transactivate the nAChR α-subunit enhancer, and (*iv*) injection of antisense myogenin oligonucleotides into BC3H-1 cells (which do not express MyoD) abolishes nAChR γ-subunit mRNA expression (37). A perplexing observation that remains is that myogenin and MyoD also bind the myosin light chain and creatine kinase enhancers, but neither gene is down-regulated by innervation. One possible explanation is that during differentiation myogenin and MyoD may modulate the expression of these genes, but after innervation their function may be substituted by other transcription factors that bind the same or different cis elements (38–40). Muscle genes that are expressed at high overall levels in innervated myofibers may be regulated by other members of the "helix-loop-helix" supergene family; members of this group of transactivating factors interact with similar cis elements (41). Another possibility is that other regulatory elements (40) could be required for the expression of these genes in innervated muscle.

Innervation has a dual component; it elicits electrical activity that regulates muscle properties (26) and provides chemical cues that may restrict the spatial distribution of molecules to the neuromuscular junction (24, 25). For example, the expression of nAChR mRNAs at extrajunctional regions of the muscle decrease dramatically during innervation, whereas the transcripts that remain are enriched at synaptic nuclei (42–45). The regional effects of nerve at the synapse may be mediated by neural-derived factors such as ARIA (24), a protein that induces expression of nAChRs. We have shown that electrical activity can down-regulate the overall levels of myogenin and MyoD mRNAs after denervation. An intriguing question that remains to be resolved is whether innervation represses the expression of myogenin and MyoD throughout most of the myofiber and leads to their localized expression at synaptic nuclei. This issue is of particular interest because it could explain how the expression of a selective group of genes is confined to the neuromuscular junction. In addition, the fact that these myogenic factors can autoregulate their own expression (46) could account for the observation that nAChR subunit mRNAs continue to be expressed preferentially in the junctional region even after denervation (45). Alternatively, longer-lasting signals that remain in the basal lamina after denervation (25) could regulate the expression of receptors, and possibly the myogenic factors. Further experiments will be needed to dissect the effects of trophic factors and electrical activity on the regulation of myogenin and MyoD expression.

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